

Type I C1 Inhibitor Deficiency with a Small Messenger RNA Resulting from Deletion of One Exon

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Abstract

The molecular genetic basis of C1 inhibitor (C1 INH) deficiency in a patient with type I hereditary angioneurotic edema was studied. This patient was found to have an abnormally short C1 INH mRNA together with a normal message. Restriction fragment length polymorphism of the C1 INH gene was detected by Southern blot analysis of the patient's DNA after digestion with Pst I or Sac I, and hybridization with a full-length C1 INH cDNA. Hybridization of the same blot with three different fragments of the full-length cDNA suggested that exon VII and portions of both flanking introns were deleted in the C1 INH gene. Northern blot analysis of RNA from cultured monocytes, using a probe corresponding to exon VII, also indicated that the abnormal C1 INH mRNA had a deletion of these nucleotides. To confirm the hypothesis that the short C1 INH mRNA contained a deletion, the involved segment of the patient's C1 INH mRNA was amplified using the polymerase chain reaction (PCR). PCR amplification yielded two C1 INH DNA fragments of different lengths (380 and 160 bp). Southern blot and sequence analysis of both DNA fragments clearly revealed that the smaller 160-bp DNA was derived from the abnormal message and had a deletion of nucleotides corresponding to exon VII.

Introduction

Hereditary angioneurotic edema (HANE)¹ is a genetic disease resulting from heterozygous deficiency of the inhibitor of the first component of complement (C1 INH) (1, 2). The disease is therefore inherited as an autosomal dominant trait, and is characterized clinically by recurrent, acute, local edema of the skin or mucosa (3, 4). Before androgen therapy for HANE was instituted (5), death from laryngeal edema was common (3, 4). The disease may be divided into two types. In type I, a normal C1 INH protein is present in plasma at reduced levels of from 5 to 30% of normal. Type II is characterized by normal-to-elevated

antigenic levels of C1 INH due to the presence of a dysfunctional mutant protein, together with low levels of the normal protein (6, 7). Previous findings, including in vitro synthesis of C1 INH by cultured monocytes (8), metabolic turnover of C1 INH (9), and analysis of restriction fragment length polymorphism (RFLP) (10) were consistent with the hypothesis that patients are heterozygous for the defect. Genetic analysis of HANE indicates that the molecular genetic defects responsible for C1 INH deficiency are heterogeneous, as are those responsible for many other genetic diseases (11–14). Analysis of mutant proteins from type II HANE patients provided the first evidence for genetic heterogeneity: the proteins from different families differ in electrophoretic mobility, size, and function (6, 15, 16). Evaluation of the C1 INH gene by Southern blot analysis in both types of HANE has revealed RFLP in ~ 10–15% of kindred (10). All of the RFLPs cosegregated with the disease and were different from one another. At least three variations in C1 INH mRNA have also been described. C1 mRNA levels in cultured monocytes from most patients with type I HANE are ~ 50% of normal (8). Three patients with markedly elevated message levels have been detected (17) and one patient with an abnormal C1 INH mRNA has been described (8). This patient had, in addition to a normal-appearing message of 2.1 kb (18), a 0.2-kb smaller mRNA that also specifically hybridized with a C1 INH cDNA probe (8).

In this study, we have analyzed the molecular genetic defect in this patient and revealed that the patient's short C1 INH mRNA resulted from a deletion of nucleotides corresponding to exon VII. This abnormality is derived from a deletion of exon VII, together with portions of both flanking introns, in the C1 INH gene.

Methods

Patient. We studied a patient with type I HANE, who has been reported previously (8). The patient had an abnormal 1.9-kb C1 INH mRNA, in addition to a normal-appearing 2.1-kb message. She had two siblings, one of whom had HANE. The sibling with HANE had the same abnormality, whereas the normal sibling had only normal appearing message. There are no other family members available for study.

Preparation of genomic DNA and Southern blot analysis. White blood cells were isolated from peripheral blood and high molecular weight genomic DNA was extracted as previously described (19). DNA was quantified by absorbance at 260 nm, and 7.5 µg of DNA was digested with restriction enzymes (Bam HI, Hind III, Pst I, Pvu II, Sac I) (New England Biolabs, Beverly, MA). DNA samples were separated by electrophoresis on 0.8% agarose gels (Bethesda Research Laboratories, Gaithersburg, MD) and blotted onto nitrocellulose (Millipore Corp., Bedford, MA) after treatment according to Wahl et al. (20). Prehybridization and hybridization were carried out at 42°C in a mixture containing 50% formamide. The C1 INH cDNAs used as probes

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1. *Abbreviations used in this paper:* C1 INH, C1 inhibitor; HANE, hereditary angioneurotic edema; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

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were as follows: a full-length probe consisting of nucleotides 1–1,810, kindly provided by Dr. Susan Bock (Temple University School of Medicine, Philadelphia, PA) and numbered according to reference 21; a probe consisting of nucleotides 1–131, derived from Bam HI digestion of the full-length probe; a probe consisting of nucleotides 1,114–1,284, derived from Kpn I/Eco RI digestion of the full-length probe; a probe consisting of nucleotides 1,289–1,810, derived from Eco RI digestion of the full-length probe. The probe 1,114–1,284 corresponds to nucleotides included within exon VII, and probe 1,289–1,810 corresponds to exon VIII (22). Labeling of the probes was achieved using the oligolabeling method (23) (Oligolabeling Kit; Pharmacia Fine Chemicals, Piscataway, NJ) using alpha [³²P]dCTP (Amersham Corp., Arlington, IL). Hybridization patterns were visualized by autoradiography.

Preparation of RNA and Northern blot analysis. Total cellular RNA was isolated from adherent monocyte monolayers (24) by lysis with guanidium thiocyanate and centrifugation through cesium chloride density gradients (25). RNA was quantitated by absorbance at 260 nm. 10 µg of total RNA was denatured with formaldehyde and formamide and separated by electrophoresis in formaldehyde-containing 1% agarose gel. Samples were transferred to nitrocellulose filters and hybridized with ³²P-labeled probes (26).

Amplification of the abnormal mRNA segment using the polymerase chain reaction (PCR) (27, 28). cDNA used for PCR was synthesized from the patient's monocyte RNA (10 µg) and from RNA derived from the hepatoma cell line, Hep G2 (29). Synthesis of cDNA followed the protocol supplied with the cDNA synthesis kit (Amersham Corp.). Two oligonucleotides for PCR primers were synthesized with DNA synthesizer (model 280B; Applied Biosystems, Foster City, CA). Both primers were 30 bases long. The primer sequences were based on nucleotide sequences on each side of the putative deletion and separated from this site by ~ 50 nucleotides. One nucleotide corresponded to nucleotides 984–1,013 in the C1 INH cDNA, and the other was complementary to nucleotides 1,335–1,364. PCR amplification of synthesized C1 INH cDNA was performed using Taq DNA polymerase following the protocol described in the DNA amplification reagent kit (Gene Amp, Perkin-Elmer Cetus, Norwalk, CT). The cycle of denaturation, annealing, and extension was repeated 25 times. Amplified cDNA was visualized on a composite gel of 3% NuSieve and 1% Sea-Kem agarose (FMC Bioproducts, Rockland, ME) (30).

DNA sequencing. Amplified cDNA was subcloned into M13 mp18 (Pharmacia Fine Chemicals) and DNA sequence analysis was carried out by the modified dideoxy chain termination method of Sanger (DNA sequencing kit; Pharmacia Fine Chemicals) (31).

Results

Southern blot analysis. RFLP of the C1 INH gene was detected on Southern blot analysis of the patient's DNA after digestion with Pst I or Sac I, and hybridization with the full-length C1 INH cDNA (1–1,810) (Fig. 1). No RFLP was observed with any of the other restriction endonucleases. The patient's DNA revealed a 6.9-kb band after digestion with Sac I and a 1.8-kb band after digestion with Pst I, neither of which was seen with DNA from control individuals. These bands were less intense than the other major bands. In addition to the diminished intensity of the polymorphic bands, one or more of the other DNA fragments in each digest appeared approximately half as intense as the equivalent band(s) from control DNA (the 2.9-kb band with Pst I digestion, the 5.8- and 3.2-kb with Sac I digestion, and the 2.7-kb with Pvu II digestion). To begin to localize the mutation responsible for the RFLP, three different fragments of the full-length cDNA were used as probes. Using probe 1–131, no RFLP was detected with any restriction enzyme and all hybridized bands were the same intensity as the equivalent bands in normal individuals (Fig. 2 A). Using probe

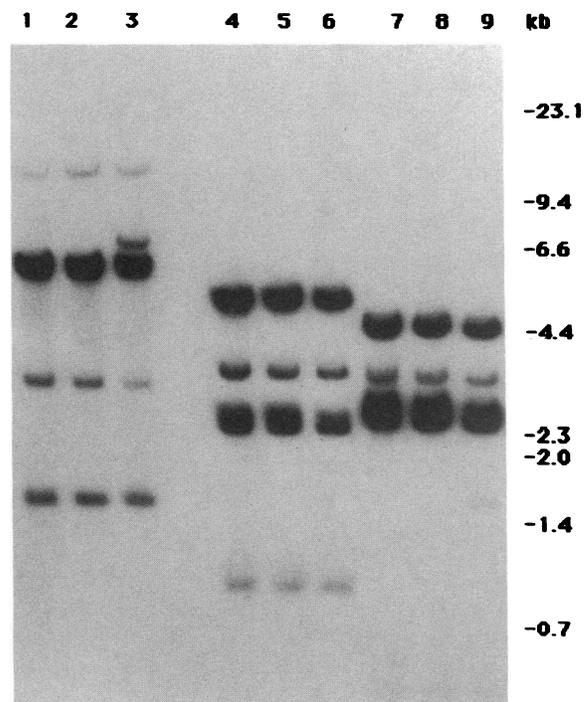


Figure 1. Southern blot analysis of high molecular weight DNA from normal individuals (lanes 1, 2, 4, 5, 7, and 8) and the patient (lanes 3, 6, and 9). DNA samples were digested with Sac I (lanes 1–3), Pvu II (lanes 4–6), and Pst I (lanes 7–9) and hybridized with the full-length C1 INH probe (1–1,810).

1,114–1,284, which is entirely included within exon VII (22), no RFLP was detected, but the hybridized bands all were less intense than the equivalent bands in normal individuals (Fig. 2 B). Hybridization with probe 1,289–1,810, which is derived from exon VIII (22), again revealed RFLP's after digestion with Pst I and Sac I (Fig. 2 C). It therefore seemed likely that the 6.9-kb Sac I fragment resulted from a mutation affecting both the 5.8- and 3.2-kb fragments (Fig. 1, lane 3, and Fig. 2, B and C, lane 2). The 1.8-kb Pst I fragment appears to be derived from the 2.9-kb band (Fig. 1, lane 9, Fig. 2, B, lane 6, and Fig. 2 C, lane 4).

Northern blot analysis. Two probes, the full-length C1 INH cDNA (1–1,810) and probe 1,114–1,284 (exon VII), were used for hybridization with Northern blots of the patient's monocyte-derived mRNA. The full-length cDNA probe hybridized with both the normal 2.1- and abnormal 1.9-kb C1 INH mRNA, as previously described (8) (Fig. 3, lane 1). Probe 1,114–1,284 hybridized only with the 2.1-kb normal C1 INH message (Fig. 3, lane 2). This suggests that the abnormal 1.9-kb mRNA results from a deletion that includes at least nucleotides 1,114–1,284.

PCR amplification of the abnormal mRNA segment. To confirm the hypothesis that the 1.9-kb C1 INH mRNA contained a deletion, the involved segment (nucleotides 984–1,364) of the patient's C1 INH mRNA was amplified using the PCR. PCR amplification of mRNA isolated from the hepatoma cell line, Hep G2, yielded a single amplified DNA band, which was ~ 380 bp in size, as expected (Fig. 4, lane 1). However, PCR amplification using the patient's mRNA resulted in the synthesis of two DNA bands (Fig. 4, lane 2). One

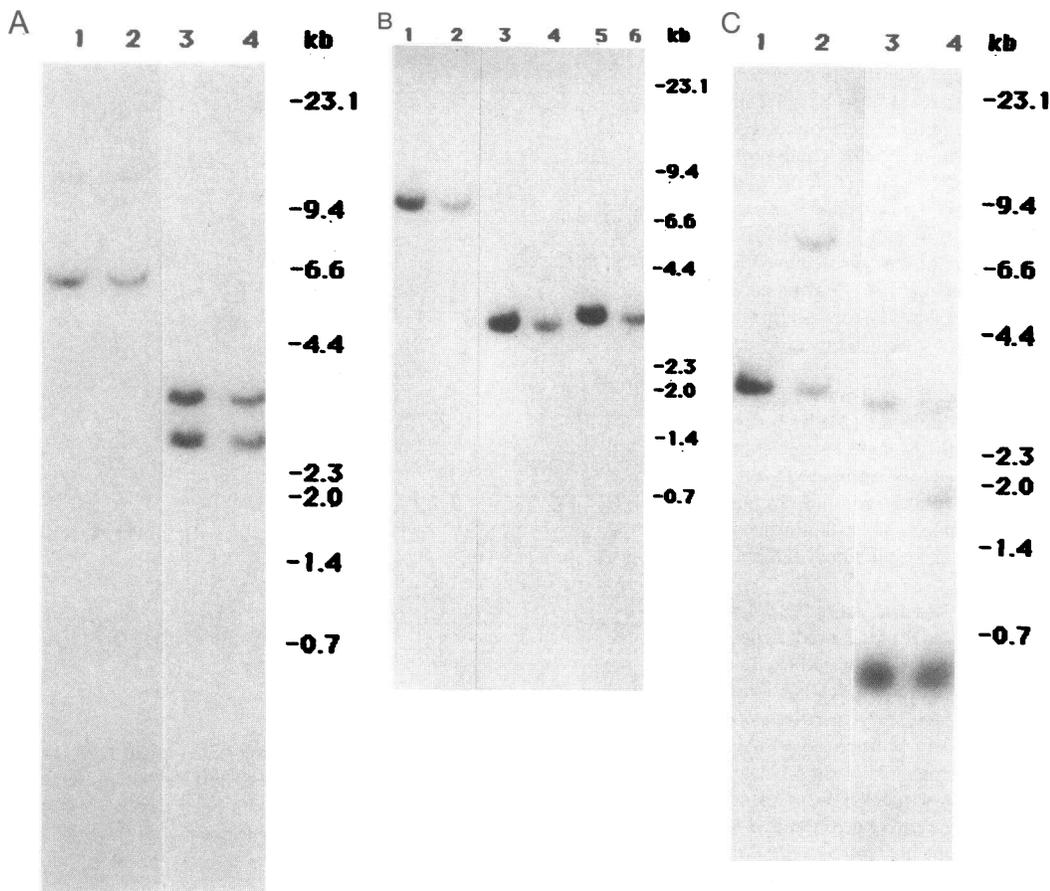


Figure 2. Southern blot analysis of DNA from a normal individual (odd-numbered lanes) and the patient (even-numbered lanes) using three different fragments of the full-length C1 INH cDNA. DNA samples were digested with Sac I (lanes 1 and 2 in A, B, and C), and Pst I (lanes 3 and 4 in A and C, and lanes 5 and 6 in B). The samples in lanes 3 and 4 in B were digested with Pvu II. No RFLP was detected after hybridization with probe 1-131 (A). Using probe 1,114-1,284, no RFLP was detected, but the hybridized bands in the patient's lanes were less intense than the equivalent bands in the normal lane (B). Using probe 1,289-1,810, the same polymorphic bands as with the full-length probe again were detected in the patient's samples.

of the DNA bands was identical in size to that derived from the Hep G2 mRNA and the other was only 160 bp long. These data further indicate that the 1.9-kb C1 INH mRNA resulted from a deletion of ~ 220 bp. Both amplified DNA fragments hybridized with the full-length C1 INH cDNA, whereas only the larger DNA hybridized with probe 1,114-1,284. (data not shown).

Nucleotide sequence analysis of the two C1 INH DNA fragments synthesized using PCR. The DNA sequence of the larger DNA was identical to the normal C1 INH cDNA from nucleotide 984 to 1364. The sequence of the smaller DNA

showed that nucleotides 1,065-1,284 were deleted (Fig. 5). These nucleotides correspond exactly to the sequence of exon VII (21, 22). There was no other abnormal nucleotide sequence in the smaller DNA. Thus, the 3' end of exon VI (nucleotide 1,064) is spliced directly to the 5' end of exon VIII (nucleotide 1,285).

Discussion

This study clearly demonstrates that this patient has a deletion that results in an abnormal, short C1 INH mRNA, in addition to a normal message. The deletion in the mRNA corresponds to exon VII (22). This abnormality almost certainly results from a deletion in the C1 INH gene that includes exon VII, rather than a splicing defect. This conclusion is consistent with the previous finding that the abnormal C1 INH mRNA in this patient was ~ 0.2-kb shorter than the normal message (exon

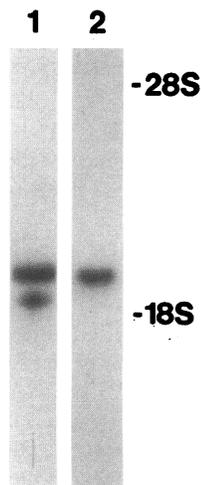


Figure 3. Northern blot analysis of RNA from the patient's monocytes. Two different sizes of C1 INH mRNA hybridized with the C1 INH full-length cDNA probe (1-1,810) (lane 1), but only the larger message (2.1 kb) hybridized with probe 1,114-1,284. 18 S and 28 S indicate the positions of 18 S and 28 S ribosomal RNA.

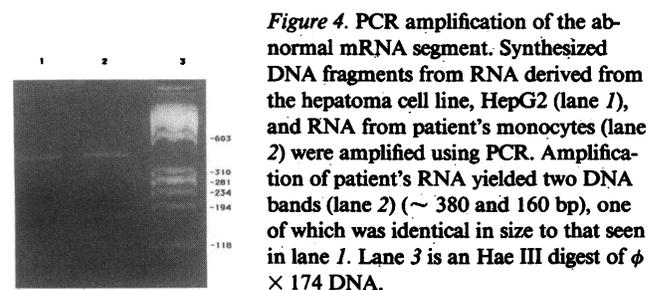


Figure 4. PCR amplification of the abnormal mRNA segment. Synthesized DNA fragments from RNA derived from the hepatoma cell line, HepG2 (lane 1), and RNA from patient's monocytes (lane 2) were amplified using PCR. Amplification of patient's RNA yielded two DNA bands (lane 2) (~ 380 and 160 bp), one of which was identical in size to that seen in lane 1. Lane 3 is an Hae III digest of $\phi \times 174$ DNA.

984
AAC TCA GTT ATA AAA GTG CCC ATG ATG AAT AGC AAG AAG TAC CCT

1029
 GTG GCT CAT TTC ATT GAC CAA ACT TTG AAA GCC AAG¹⁰⁶⁴AAT TCT TCG

1294
 ATT TTT CTT ATG ACC TTA ACC TGT GTG GGG TGA CAG AGG AG^{*} CAG

1339
ATC TTC AGG TTT CTG CGA TGC AGC AC

Figure 5. Sequence analysis of the 160-bp DNA fragment synthesized using PCR. Nucleotides 1,065–1,284 of the normal cDNA were deleted. The splice site between nucleotides 1,064 and 1,285 is indicated by the dagger. The premature termination codon created by the frame shift resulting from the deletion is indicated by the asterisk. The sequences used for construction of oligonucleotide primers for PCR are underlined.

VII is 220 bp long). From the limited information currently available, partial deletions within the C1 INH gene probably account for 15–20% of families with type I HANE (10, 17, 32, 33). It is possible, as suggested by Carter et al. (22), that these deletions are related to the presence of Alu I repeats (34) within some of the introns in the C1 INH gene. These repeats are thought to predispose to deletions and insertions as a result of unequal crossing over. The deletion described here is consistent with this explanation because Alu I repeats are present at least in the intron preceding exon VII (22). Sequence analysis of genomic clones of the abnormal gene from this patient and of the intron after exon VII in the normal gene will be required to confirm this suggestion.

Southern blot analysis after digestion with Pst I and Sac I detected RFLP using the full-length C1 INH cDNA as a probe. All bands that hybridized with probe 1,114–1,284 were about half the intensity of the equivalent bands from control individuals. These results strongly suggest that one of the C1 INH alleles in this patient has a deletion that includes at least the nucleotides corresponding to this probe, which is contained within exon VII. As described in Results, the 6.9-kb polymorphic fragment seen in a Sac I digest of the patient's DNA, is probably derived from the 5.8- and 3.2-kb Sac I fragments. This suggests that, if the polymorphism is the result of a deletion, this deletion must be ~ 2 kb long. This hypothesis must, however, be confirmed by sequence analysis of appropriate C1 INH genomic clones from the patient. RFLPs were again observed using probe 1,289–1,810. This probe is contained entirely within exon VIII (22). Because it detects an RFLP, this suggests that it is outside, but near, the deletion. The 3' end of the deletion, therefore, does not extend through exon VIII. A genomic DNA library has been constructed from this patient's DNA, and clones corresponding to both the normal and abnormal C1 INH alleles have been isolated. Preliminary analyses of these clones are consistent with the above hypothesis. Southern blot analysis and detailed restriction mapping confirm that the abnormal allele contains no DNA corresponding to exon VII and that the deletion is ~ 2 kb in length (unpublished data).

The patient's abnormal C1 INH mRNA that hybridized with full-length C1 INH cDNA probes, did not hybridize with probe 1,114–1,284, indicating that at least this segment of exon VII was deleted. Using this information, oligonucleotide primers for PCR were constructed to amplify the sequence

between nucleotides 984 and 1,364. Using the patient's mRNA, this resulted in two DNA fragments, one of which was ~ 380 bp long, as would be predicted for the normal message, whereas the other was 220 bp shorter. Sequence analysis of these amplified DNA fragments confirmed that the shorter one had a deletion that corresponded to exon VII. Thus, although the patient probably has a deletion in the C1 INH gene that includes exon VII (and portions of both flanking introns), transcription and RNA processing apparently proceeded normally to produce the small mRNA.

The patient has type I HANE. Although she has an abnormal C1 INH mRNA, no abnormal C1 INH protein has been detected in her serum (8). In addition, no abnormal C1 INH protein was synthesized by cultured monocytes from this patient (8). Intracellular C1 INH levels in these cultured monocytes were ~ 50% of normal, as were the intracellular C1 INH levels in monocytes from the other type I patients. A pulse-chase experiment with this patient's monocytes revealed no difference in the kinetics of synthesis or secretion of C1 INH as compared with other type I patients or with normals (8). Thus, no truncated protein that might result from translation of the abnormal mRNA has been detected, and there is no apparent abnormality in transcription or translation of the normal allele. Deletion of the nucleotides corresponding to exon VII in the abnormal C1 INH mRNA created a frame-shift resulting in a premature termination codon at the 14th triplet after the deletion (Fig. 5). There are at least three potential explanations for the inability to detect an abnormal C1 INH protein in her serum. It is possible that the abnormal mRNA is not translated. If translation occurs, a prematurely terminated protein would result (334 residues compared with 478 residues for the normal protein). It is very likely that this polypeptide would be rapidly degraded intracellularly. In vitro translation experiments will be required to answer these questions. Finally, it is possible that the mRNA itself is unstable. Northern blot analysis is consistent with this possibility. Although the original preparations of RNA from the patient's monocytes revealed equal amounts of normal and small C1 INH mRNA, most subsequent preparations have shown diminished quantities of the smaller message (Fig. 3).

It is noteworthy that the abnormally short C1 INH mRNA in this patient is detectable, although the amount is less than the normal message. Mutations that cause premature termination usually are associated with a marked quantitative decrease in the amount of specific mRNA (35, 36). It has been suggested that this kind of mutation may affect intranuclear RNA stability or nuclear to cytoplasmic transport (37, 38). Although abnormal specific mRNA has been associated with some genetic diseases, most mutations described have been point mutations or some other mutation that affects the RNA splicing process. Such abnormal mRNAs usually have been detectable only by analyses such as S₁ nuclease protection assays or sequence analysis (39, 40). Yang et al. reported an abnormally large message in a patient with hypoxanthine-guanine phosphoribosyl transferase deficiency due to gene duplication (38). In instances of deletions large enough to include an entire exon, mRNA usually is not detected (12, 41, 42). Therefore, further analysis of the abnormality in this patient, including the sequence of the relevant portion of the abnormal C1 INH gene, and in vitro expression studies, may shed light on the requirements for normal transcription and translation.

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